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High surface adsorption properties of carbon-based nanomaterials are responsible for mortality, swimming inhibition, and biochemical responses in *Artemia salina* larvae

Tina Mesarič[a], Chiara Gambardella[b], Tamara Milivojević[a], Marco Faimali[b], Damjana Drobne[a,c,d], Carla Falugi[e], Darko Makovec[f], Anita Jemec[a], Kristina Sepčić[a,*]

[a] *Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia*
[b] *Institute of Marine Sciences, National Research Council, Genova, Italy*
[c] *Centre of Excellence in Nanoscience and Nanotechnology (CO Nanocentre), Ljubljana, Slovenia*
[d] *Centre of Excellence in Advanced Materials and Technologies for the Future (CO NAMASTE), Ljubljana, Slovenia*
[e] *Department of Earth, Environment and Life Sciences, University of Genova, Genova, Italy*
[f] *Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia*

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ABSTRACT

We investigated the effects of three different carbon-based nanomaterials on brine shrimp (*Artemia salina*) larvae. The larvae were exposed to different concentrations of carbon black, graphene oxide, and multiwall carbon nanotubes for 48 h, and observed using phase contrast and scanning electron microscopy. Acute (mortality) and behavioural (swimming speed alteration) responses and cholinesterase, glutathione-S-transferase and catalase enzyme activities were evaluated. These nanomaterials were ingested and concentrated in the gut, and attached onto the body surface of the *A. salina* larvae. This attachment was responsible for concentration–dependent inhibition of larval swimming, and partly for alterations in the enzyme activities, that differed according to the type of tested nanomaterials. No lethal effects were observed up to 0.5 mg/mL carbon black and 0.1 mg/mL multiwall carbon nanotubes, while graphene oxide showed a threshold whereby it had no effects at 0.6 mg/mL, and more than 90% mortality at 0.7 mg/mL. Risk quotients calculated on the basis of predicted environmental concentrations indicate that carbon black and multiwall carbon nanotubes currently do not pose a serious risk to the marine environment, however if uncontrolled release of nanomaterials continues, this scenario can rapidly change.

1. Introduction

Naturally occurring and industrially derived carbon-based nanomaterials are one of the most abundant nanomaterials in the environment (Hussain et al., 2010). However, some carbon-based nanomaterials have potentially toxic effects. It is therefore important to understand their effects on the biota, especially of aquatic environments, where they can finally accumulate.

Carbon black (CB) is a form of amorphous carbon with extensive uses in industrial applications (e.g. rubber products, paint, plastic, ink). It is manufactured by controlled vapour-phase pyrolysis of hydrocarbons (Sorahan and Harrington, 2007; Screening Assessment for the Challenge, 2013), and it should not be confused with black carbon, which is a product of incomplete combustion of fossil fuels and biomass (Goldberg, 1985; Masiello and Dreffel, 1998). Graphene oxide (GO) is a single planar layer of carbon atoms that is arranged into a honeycomb-like lattice (Norwegian Pollution Control Authority, 2008). This has recently attracted great interest for its potential applications in electronics, energy, materials and biomedical areas. Carbon nanotubes (CNTs) are 'rolled-up' graphene sheets that can be capped or not at both ends by a half fullerene (another allotrope of carbon). CNTs can be formed of single (single-wall CNTs; SWCNTs) or multiple

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Abbreviations: CB, carbon black; GO, graphene oxide; FNSW, filtered natural sea water; MWCNTs, multiwall carbon nanotubes; PEC, predicted environmental concentration; PNEC, predicted no-effect concentration; RQ, risk quotient; SEM, scanning electron microscopy.

* Corresponding author at: Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia. Tel.: +386 1 3203419; fax: +386 1 257339.

E-mail addresses: tina.mesaric84@gmail.com (T. Mesarič), chiara.gambardella@ge.ismar.cnr.it (C. Gambardella), milivojevictamara@gmail.com (T. Milivojević), marco.faimali@ismar.cnr.it (M. Faimali), damjana.drobne@bf.uni-lj.si (D. Drobne), carlafalugi@hotmail.it (C. Falugi), darko.makovec@ijs.si (D. Makovec), anita.jemec@bf.uni-lj.si (A. Jemec), kristina.sepcic@bf.uni-lj.si (K. Sepčić).

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(multiwall CNTs; MWCNTs) carbon layers (Norwegian Pollution Control Authority, 2008). With their unique electrical, thermal, chemical, mechanical and optical properties, CNTs are one of the most interesting, prospective and used nanomaterials (Park and Ruoff, 2009).

Most of the available data on the toxicity of carbon-based nanomaterials towards aquatic invertebrates have been reported for freshwater model crustaceans, such as *Daphnia magna* (Arndt et al., 2013a; Baumerte et al., 2013; Lovern and Klaper, 2006; Oberdörster et al., 2006; Petersen et al., 2009; Roberts et al., 2007), *Daphnia pulex* (Klaper et al., 2009), *Ceriodaphnia dubia* (Li and Huang, 2011) and *Hyallela azteca* (Kennedy et al., 2008). These studies have provided evidence that acute and chronic exposure to carbon-based nanomaterials at concentrations from 0.4 mg/mL up to more than 35 mg/mL can cause adverse effects for aquatic organisms. Some studies on carbon-based nanomaterials have also been carried out on marine organisms; e.g. crustaceans, bivalves and polychaetes. Canesi et al. (2010a,b); Canesi et al. (2010a,b) reported adverse effects of CB and C_{60} fullerenes (0.05–10 μg/mL) on *Mytilus galloprovincialis* after 24 h of exposure. The C_{60} fullerenes and CNTs (1–10 μg/mL) were also shown to induce cytotoxicity in immune cells of *M. galloprovincialis* (Moore et al., 2009). Effects of CB and MWCNTs on the marine crustacea *Artemia salina* after 48 h of exposure were studied by Baumerte et al. (2013), with reported LC_{50} of 0.03 mg/mL and 0.013 mg/mL, respectively. Miglietta et al. (2011) also showed that these two nanomaterials can be toxic for *A. salina*. In their study, exposure of *A. salina* to suspensions of CB and MWCNTs (66 μg/mL) induced mortalities of 50% and >95%, respectively, after only 24 h. In a study by Pretti et al. (2014), no toxicity of different types of pristine graphene to *A. salina* was recorded up to 10 mg/L after a 24 h of exposure. However, after a 48 h exposure, pristine graphene monolayer flakes up to the concentration of 1 mg/L increased the biomarkers of oxidative stress (catalase and glutathione peroxidase activity), as well as the levels of lipid peroxidation. Templeton et al. (2006) showed an increase in mortality and a delay in development for the marine copepod *Amphiascus tenuiremis* after a 35-day exposure to 10 μg/mL SWCNT. The recent study by Sohn et al. (2015) reveals that SWCNTs have no short-term acute toxicity against *D. magna* up to 100 μg/mL, but they can affect freshwater microalgae. On the other hand, carbon-based NMs including SWCNT and MWCNT were found to exert a multigenerational effect on *D. magna* survival, reproduction, and growth as exposure of the parent population (Arndt et al., 2013b). In our previous study (Mesarič et al., 2013), we reported adverse effects of GO and CB (up to 1 and 5 mg/mL, respectively) on the behaviour and survival of the barnacle *Amphibalanus amphitrite* after 24 h and 48 h of exposure.

The literature data have reported high surface adsorption potential of carbon-based nanomaterials in comparison with metal-based ones (Ruh et al., 2012; Xia et al., 2011; Li et al., 2013). This potential is postulated to be the primary driving force behind all of the interactions and dynamic changes between nanomaterials and biological systems (Xia et al., 2011). Therefore, it can be expected that compounds with high surface-adsorption potential will strongly attach onto the surface of aquatic invertebrates, and will affect their activities, like swimming, filtration, breading and moulting. In the present study, we selected three different carbon-based nanomaterials and studied their effects on the larvae of the crustacean *A. salina*: CB, GO, MWCNTs. This organism is a suitable and widely used model organism in studies of acute toxicity (Ates et al., 2009). Here, we measured the different ecotoxicological end-points of mortality and behavioural response (swimming speed alteration), and some selected biochemical biomarkers that are frequently analysed in toxicological studies, as the activities of the cholinesterase, glutathione-S-transferase and catalase enzymes (Boldina-Cosqueric et al., 2010; Buffet et al., 2011; Garaventa et al., 2010).

We hypothesised that the high surface-adsorption potential of these carbon-based nanomaterials might be the main factor that is responsible for the expected responses (i.e. mortality, alteration of enzyme activities), and would also affect the swimming behaviour of this model organism. We aimed to use the data from this study for environmental risk assessment of carbon-based nanomaterials in marine environments, by assessing the ratio between their predicted environmental concentration (PEC) and predicted no-effect concentration (PNEC).

2. Material and methods

2.1. Characteristics of carbon-based nanomaterials

Carbon black was purchased from PlasmaChem GmbH (Germany). The mean size of the primary particles was estimated from transmission electron microscopy (TEM) analysis (TEM Jeol 2010 F operated at 200 kV) to be 13 nm. Single-layer GO was from Graphene Supermarket, as dry flakes with an average size of 0.5–5μm, as determined from the TEM. At least 80% of the GO was composed of single-layer molecules. MWCNTs were obtained through the EU FP7 large-scale integrating project known as NanoValid and were supplied by Nanocyl (http://www.nanocyl.com/). According to the TEM analysis the MWCNTs had an outer diameter ranging from 5.7 to 15 nm and were several μm long (Supplementary information Fig. S1).

All three carbon-based nanomaterials were freshly prepared as suspensions in dH_2O to provide stock suspensions at the final concentration of 1 mg/mL, which were sonicated for 30 min in an ultrasonic bath sonicator (model LBS1, Falc; Italy), as described in Canesi et al. (2010a). The stock dispersions of nanomaterials were further diluted to toxicity test concentrations in FNSW.

2.1.1. Characteristics of the carbon-based nanomaterials in distilled water

ζ-Potential of the carbon-based nanomaterials dispersed in distilled water (0.01 mg/mL) was measured using a Brookhaven Instruments Corp., ZetaPALS. In distilled water, the carbon-based nanomaterials showed a relatively high, negative ξ-potential at neutral pH (−32 mV, −36 mV, and −21 mV for CB, GO, and MWCNT, respectively). Although the ξ-potential of the CB suspension exceeded the absolute value of 30, which is considered to be high enough to electrostatically prevent the particles from agglomeration (Tadros, 1987); substantial agglomeration was observed by dynamic light scattering. The size of the CB agglomerates in the distilled water suspension ranged between 60 nm and 170 nm, and around 300 nm (Mesarič et al., 2013). The dynamic light scattering analyses of the GO and MWCNT suspensions were not possible because of the extremely anisotropic shape of the primary particles.

2.1.2. Characteristics of the carbon-based nanomaterials in FNSW

The ξ-potential (Brookhaven Instruments Corp., ZetaPALS) of nanomaterials suspended in the FNSW is much lower to that in the distilled water because of electrostatic screening effects related to the very high ionic strength. The ξ-potentials of approximately −14 mV were measured for the all three types of carbon-based nanomaterials in the FNSW. Due to the decreased effective electrostatic repulsions between the particles in the FSNW they quickly flocculate. Under strong mechanical shearing, i.e. during sonication, the soft floccules brake into smaller ones. However, when the sonicator is turned off, the floccules grow to the size visible to a naked eye in just few minutes. This is also the reason why reliable measurements of the hydrodynamic size of the FNSW-suspended nanomaterials using dynamic light scattering were not possible,